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ONCOFETAL ANTIGEN IN *XIPHOPHORUS* DETECTED BY MONOCLONAL ANTIBODIES DIRECTED AGAINST MELANOMA-ASSOCIATED ANTIGENS

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Monoclonal antibodies (MAbs) directed against *Xiphophorus* melanoma cells were developed and tested by indirect immunofluorescence and immunoperoxidase staining for reactivity with a panel of 15 allogeneic tissues and 12 allogeneic cell lines. The reactivity of such MAbs was restricted to melanoma cells from tumor biopsies and melanoma-derived cell lines. In addition, all embryonic cells of all histiotypes from developmental stages later than mid-organogenesis and from corresponding short term *in vitro* cultures reacted with these MAbs. In contrast, normal tissues and organs from adult fish displayed no reactivity, thus implying that the melanoma-associated antigens detected by the MAbs described are oncofetal antigens.

In mammalian melanoma evidence for a specific reaction of the host immune system to the autologous tumor is 2-fold: (a) at the humoral level by the presence of autologous anti-melanoma antibodies in sera of melanoma patients (Albino *et al.*, 1981), and (b) at the cellular level by cytotoxic leukocytes infiltrating the neoplastic tissue (Rosenberg *et al.*, 1988).

The ability of the immune system to discriminate between neoplastically transformed cells and their normal counterparts indicates that melanoma cells carry determinants on their surface which are distinct from those of the corresponding normal pigment cells. These determinants are, therefore, called melanoma-associated antigens (MAAs).

Such structures have been identified on human primary melanoma and on *in vitro* cultured melanoma cell lines (Koprowski *et al.*, 1978; Yeh *et al.*, 1979; Dippold *et al.*, 1980; Morgan *et al.*, 1981; Wilson *et al.*, 1981; Carrel *et al.*, 1982; Reisfeld *et al.*, 1982; Hellström *et al.*, 1983; Herlyn *et al.*, 1983; Brüggen *et al.*, 1984; Holzmann *et al.*, 1985; Kan-Mitchell *et al.*, 1986; Natali *et al.*, 1987). Furthermore, Taniguchi and Wakabayashi (1964) have demonstrated interspecies conservation of MAAs that are shared by melanoma cells of hamster, mouse and humans. Although MAAs offer unique possibilities for tumor diagnosis and therapy in humans, little is known about their biological role and the salient feature of their structural heterogeneity. As questions of this kind are better approached in genetically defined experimental systems, we are attempting to utilize the melanoma system of the poeciliid fish *Xiphophorus* (Anders *et al.*, 1984). Pigment-cell neoplasia in the teleost fish *Xiphophorus* became a useful system with which to study the changes underlying and accompanying the process of tumor formation. The causative melanoma-inducing gene has been cloned and characterized (Wittbrodt *et al.*, 1989).

The melanoma of humans and *Xiphophorus* have several structural and pathological features in common, such as (a) morphological and ultrastructural similarities (Sobel *et al.*, 1975; Riehl *et al.*, 1984), (b) progressive growth of melanoma in humans (Brüggen *et al.*, 1981) and fish (Anders *et al.*, 1979), (c) tumor regression in humans (Thompson, 1973) and fish (Anders and Anders, 1978), (d) the ability of human (Herlyn *et al.*, 1985) and fish melanoma (Peter *et al.*, 1985; Schartl and Peter, 1988) to grow in thymus-aplastic nude mice, (e) similarities between the ganglioside component profiles of melanoma cell surfaces in human and fish (Felding-Habermann *et al.*, 1988), and (f) expression of the c-*src* oncogene in fish and human melanoma (Schartl *et al.*, 1985; Barnekow *et al.*, 1987). Hereditary factors have been shown to be responsible for the spontaneous development of melanoma in *Xiphophorus* (Anders *et al.*, 1985), and in some human melanoma there is also evidence that genetic factors might contribute to the etiology (Rhodes *et al.*, 1983; Lynch *et al.*, 1985). A major drawback for the usefulness of this experimental system for comparative studies is the lack of any information relating to tumor immunology. As a first step in order to demonstrate and to characterize MAAs in *Xiphophorus* we have produced MAbs against cell-surface antigens of fish melanoma cells by the somatic cell fusion technique (Köhler and Milstein, 1975). Here we report on MAAs in *Xiphophorus* detected by those MAbs and we present evidence that these MAAs are oncofetal antigens.

MATERIAL AND METHODS

Experimental animals

The fish used in this study were bred under standard conditions (Kallman, 1975) in the aquarium of the Genetics Institute at the University of Giessen and the Gene Center of the Max-Planck-Institute for Biochemistry in Martinsried. Crossings (Fig. 1) between *X. maculatus* (A) and *X. helleri* (B) result in the F_1 generation (C), that develops benign melanoma in the dorsal fin. Back-crossing of these hybrids with *X. helleri* (B) produces 3 different types of segregant: 25% of the offspring develop benign melanoma (D), 25% develop malignant melanoma (E) and 50% of the animals are tumor-free (F). A detailed description of the crossing procedures, genotypes and phenotypes is given by Anders *et al.* (1973, 1981). In addition, albino fish carrying amelanotic malignant melanoma were used (corresponding to genotype E).

Brain, muscle, heart, spleen, liver, testes, gut, gill, kidney and skin from non-tumorous fish of genotype F as well as melanoma and melanoma-free tissue of fish of genotype E were resected and immediately processed to cell suspensions for indirect immunofluorescence (IF) or to cryostat sections for immunoperoxidase staining (IP). Tumor-free embryos of *Xiphophorus* hybrids (genotype F) were staged according to Tavolga (1949) and processed to cell suspensions or short-term cultures.

In addition, one carcinogen-induced rhabdomyosarcoma from a *Xiphophorus* hybrid (kindly provided by Dr. C.R. Schmidt, Giessen) and tumor cells from a melanotic melanoma derived from fish of genotype E and passaged through a nude mouse (Schartl and Peter, 1988) were used.

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Abbreviations: MAA(s), melanoma-associated antigen(s); MAb(s), monoclonal antibody(ies); IF, indirect immunofluorescence; IP, immunoperoxidase staining.

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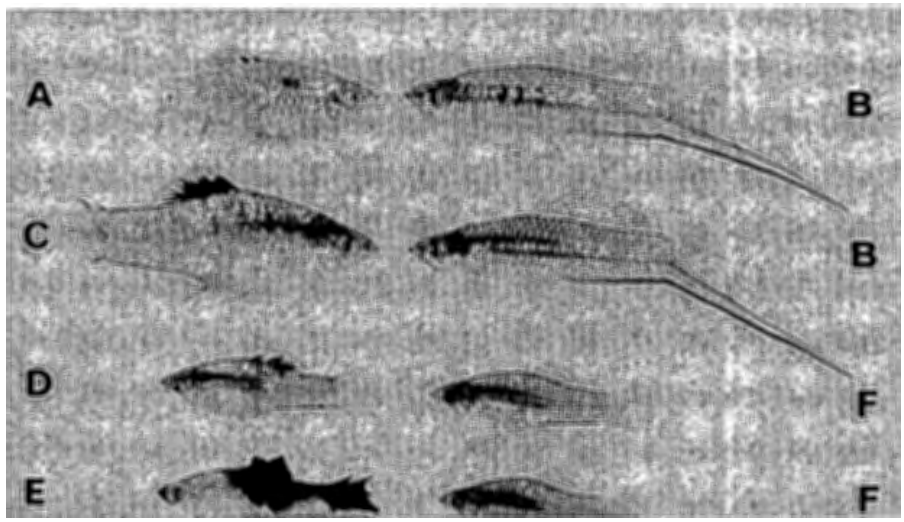

FIGURE 1 – Crossing scheme of the platyfish (*X. maculatus;* A) and the swordtail (*X. helleri;* B); (C) F_1-hybrid and (D) BC_1 hybrid developing benign melanoma; (E) BC_1-hybrid developing malignant melanoma; (F) melanoma-free BC_1 segregants; for explanation see text.

Cell lines and in vitro *culture conditions*

Cells of the non-secreting, 8-azaguanine-resistant mouse myeloma line P3X63-Ag8.653 (Kearney *et al.*, 1979), used as a fusion partner for the mouse spleen cells, as well as the hybridoma cells, were grown in RPMI 1640 containing 10% FCS, 1mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (GIBCO, Karlsruhe, FRG) at 37°C with 5% CO_2.

Cell cultures used to study the reactivity pattern of the MAbs were derived from (a) hereditary melanoma of *Xiphophorus* hybrids comparable to genotype E (PSM, amelanotic under normal culture conditions) (Wakamatsu, 1981), (b) melanoma induced by ethyl- or methyl-nitrosourea (97, ArSr4, E18), (c) non-tumorous embryos of wild-type *X. xiphidium* (XX, A2), (Kuhn *et al.*, 1979), wild-type *X. helleri* (224, HIII), wild-type *X. maculatus* (781, Bst) or from backcross hybrids, genotype F (BC, 472).

Established fish cell lines were cultured in F12 medium supplemented as mentioned above but incubated at 28°C with 5% CO_2.

For primary cultures, 20–30 embryos were washed in sterile PBS (8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na_2HPO_4, 0.132 g/l $CaCl_2$, 0.2 g/l KH_2PO_4, 0.1 g/l $MgCl_2$) containing 500 IU/ml penicillin, 500 μg/ml streptomycin and 5 μg/ml fungizone. They were subsequently minced into small pieces and incubated with 0.05% trypsin/0.02% EDTA for 2 hr at room temperature. The cell suspension was centrifuged for 10 min at 200 *g*, washed with PBS, and resuspended in tissue culture medium.

Generation of MAbs

BALB/c mice were immunized intraperitoneally with a suspension of 2–5 × 10^7 *Xiphophorus* melanoma cells in HBSS (8 g/l NaCl, 0.4 g/l KCl, 0.1 g/l $MgSO_4$, 0.048 g/l Na_2HPO_4, 0.185 g/l $CaCl_2$, 0.35 g/l $NaHCO_3$, 0.06 g/l KH_2PO_4, 0.1 g/l $MgCL_2$, 1 g/l glucose) 3 times at 2-weekly intervals. Four days after the last immunization, the spleen cells were fused with P3X63-Ag 8.653 mouse myeloma cells at a 1:2 ratio using standard procedures (Fazekas de St Groth and Scheidegger, 1980). Fused cells were resuspended in complete RPMI 1640 culture medium containing 0.1 mM hypoxanthine, 16 μM thymidine and 0.4 μM aminopterin (HAT selection medium, GIBCO) and plated with a density of 2 × 10^4 per well in 24-well plates containing 2 × 10^4 mouse peritoneal cells as a feeder layer. Hybrid supernatants were screened for antibodies recognizing the primary fish melanoma. Positive hybridomas were cloned twice by limiting dilution (Fazekas de St Groth and Scheidegger, 1980).

Immunological studies

Tissue culture supernatants were used as a source of MAbs in the immunological studies. For this purpose MAbs 21-7 and 4-7-2 were cultured in serum-free medium (KC 2000, KC Biologicals, Leuaxa, KS) while this was not possible for MAb 2-18. The reactivity of the MAbs with cell-surface antigens was analyzed by IF as described by Natali *et al.* (1981). IP staining of cytocentrifuge preparations of cell suspensions and cyrostat sections of tissues was performed basically as described by Lowenthal *et al.* (1985).

Metabolic labelling of cells and immunoprecipitation

Radiolabelling, immunoprecipitation and SDS-PAGE were performed essentially as described by Mitchell *et al.*, (1980) except that ^{35}S-methionine was used for overnight metabolic labelling of the cells. For size calibration a ^{14}C-labelled protein standard was used containing myosin (200 kDa), phosphorylase (92.5 kDa), bovine serum albumine (69 kDa), ovalbumine (46 kDa), carboanhydrase (30 kDa), lysozyme (14.3 kDa).

RESULTS

Three fusion experiments of spleen cells from mice immunized with cells of malignant melanotic melanoma from *Xiphophorus* yielded 445 hybridomas. The supernatants of 5 of these contained antibodies which recognized fish melanoma cells in suspension. The corresponding hybridomas were cloned by limiting dilutions in order to generate MAbs. Three out of the 5 MAbs—designated 21-7, 4-7-2 and 2-18—were selected for further studies. MAbs 21-7 and 4-7-2 were typed as belonging to the IgG_1-subclass, MAb 2-18 was found to be a IgG_{2b} isotype (data not shown). The 3 MAbs were tested for reactivity on different normal adult tissues, on embryonic cells and on cells of a panel of *in vitro* cultures. The IF and the IP techniques (see "Material and Methods") were applied for the detection of surface-membrane bound epitopes, and for recognition of cytoplasmic antigens, respectively.

TABLE I – REACTIVITY PATTERN OF THE MAbs DEVELOPED AGAINST FISH MELANOMA CELLS WITH DIFFERENT FISH TISSUES, TESTED BY INDIRECT IMMUNOFLUORESCENCE (IF) AND INDIRECT IMMUNOPEROXIDASE (IP) ASSAY

	Reactivity of the anti-fish melanoma MAb in					
	IF			IP		
	21-7	4-7-2	2-18	21-7	4-7-2	2-18
Hereditary melanotic melanoma	+(30%)[1]	+(30%)	+(50%)	+(40%)	+(40%)	+(60%)
Hereditary amelanotic melanoma	NT	NT	NT	+(100%)	+(100%)	+(100%)
Melanoma transplant in nude mouse	+(50%)	+(50%)	+(50%)	NT	NT	NT
Induced rhabdomyosarcoma	—	—	NT	—	—	NT
Tissues of non-tumorous fish (liver, testes, spleen, kidney, brain, skin, gill, gut, muscle, heart)	—	—	—	—	—	—
Healthy tissue of melanoma-bearing fish	NT	NT	NT	—	—	—

[1]Fraction of positive reacting cells out of approximately 200 cells counted; + = positive, — = negative. NT = not tested.

When cell suspensions were used, all 3 MAbs reacted with structures of the primary fish melanoma (Table I). These structures were predominantly present on the cell surface. Moreover, the 3 MAbs showed reactivity with the fish melanoma transplant passaged through a nude mouse. The structures recognized by MAb 21-7 and 4-7-2 were not present on a tumor of a different histiotype, fish rhabdomyosarcoma. No binding could be detected with cells from cell suspensions from different organs of normal fish or from non-tumorous organs of melanoma-bearing fish. Most importantly, normal pigment cells did not stain with any of the 3 MAbs by IF or IP.

Table II shows that MAbs 21-7 and 4-7-2 but not MAb 2-18 recognized antigens present on the surface of the PSM melanoma cells, whereas all 3 MAbs reacted with the cell lines derived from chemically induced melanoma. Antibody binding did not occur if the melanoma cells were treated with 0.05% trypsin for 10 min prior to incubation with MAb. Treatment with 0.02 IU/ml neuraminidase for 30 min did not interfere with the antibody reaction (data not shown). This indicates that the antigenic determinants recognized by the MAbs are protein structures on the cell surface. To further characterize the MAAs recognized by MAb 4-7-2 and 21-7, PSM melanoma cells were metabolically labelled. Separation of the immunoprecipitated proteins by SDS-PAGE consistently revealed a protein duplet of approximately 130 and 135 kDa with either MAb 4-7-2 or MAb 21-7 (Fig. 2). No such protein was precipitated in controls with MAbs directed against human MAAs not present in fish cells.

To study the distribution of the antigens *in situ*, histological sections of amelanotic melanoma from albino fish were used to avoid problems arising from the high melanin content of melanotic melanoma that obscures cellular and subcellular structures. All 3 MAbs reacted only with the tumor tissue, while the surrounding healthy tissue remained unstained (Fig. 3). Studies on the ontogenetic expression pattern of MAb 21-7 and 4-7-2 revealed that they detect antigens on the surface of embryonic cells (Table III, Fig. 4). These antigens were present on all embryonic cells, regardless of their histiotype, starting from mid-organogenesis stages (stage 10, Tavolga, 1949), but disappeared in neonates over 4 days old. If embryonic cells (from total embryos of stage 25) were maintained *in vitro* for more than 4 weeks without subculturing, the antigen disappeared from the cell surface, as tested by MAb 4-7-2 at weekly intervals up to 10 weeks (data not shown). This was also observed in continuously passaged cell cultures derived from embryos (Table II). In all second-passage cultures a certain percentage of cells (5%–80%) still reacted with all 3 MAbs. In later passages, however, 4 of the 5 cell lines tested showed no reactivity at all with MAbs 21-7 and 4-7-2. Only about 30% of the cells from cell line 472 still expressed these antigens. MAb 2-18 reacted with all second-passage cell cultures and established cell lines tested so far.

The antigenic determinants detected by MAb 21-7 and 4-7-2 expressed on the surface of melanoma cells and embryonic cells but not on normal adult cells are therefore considered to be oncofetal antigens.

DISCUSSION

We have presented evidence of oncofetal antigens in the fish *Xiphophorus*. They were identified by 2 MAbs (21-7, 4-7-2) on the surface of embryo cells starting from mid-organogenesis stages and on the surface of melanoma cells. In contrast, they were not found on 10 different organs derived from non-tumorous fish or on non-tumorous tissue of melanoma-bearing animals. This result indicates that these antigens are not identical with histocompatibility antigens, which have also been identified in *Xiphophorus* (Kallman, 1964). The antigens recognized by the MAbs are absent on *Xiphophorus* rhabdomyo-

TABLE II – REACTIVITY PATTERN OF THE MAbs DEVELOPED AGAINST FISH MELANOMA WITH CULTURED FISH CELLS OF DIFFERENT ORIGIN, AS TESTED BY INDIRECT IMMUNOFLUORESCENCE (IF)

Cell line	Origin	Reactivity of the MAb in IF		
		21-7	4-7-2	2-18
PSM	Hereditary fish melanoma	+(100%)[1]	+(100%)	—
97	Carcinogen-induced melanoma	+(30%)	+(40%)	+(50%)
ArSr		+(30%)	+(10%)	+(90%)
E18		+(40%)	+(80%)	+(100%)
781	Second passage of primary cell cultures of embryos	+(10%)	+(60%)	+(80%)
H III		+(10%)	+(10%)	+(20%)
BC		+(5%)	+(10%)	+(40%)
A2	Established embryonic cell lines	—	—	+(100%)
XX		—	—	+(10%)
224		—	—	NT
Bst		—	—	NT
472		+(30%)	+(30%)	NT

[1]Fraction of positive reacting cells out of approximately 200 cells counted; + = positive, — = negative. NT = not tested.

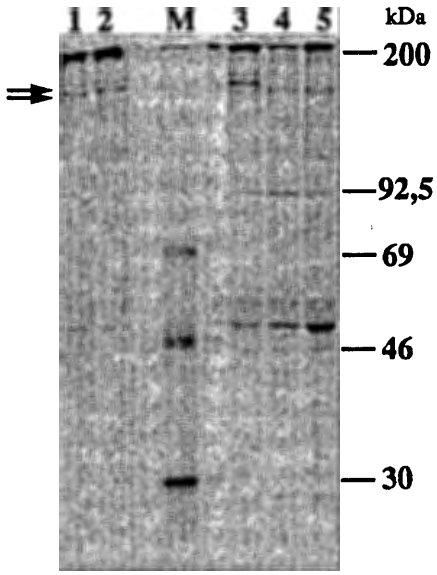

FIGURE 2 – Immunoprecipitations from metabolically labelled cell extract of PSM melanoma cells with MAbs 21-7 (lanes 1, 2) and 4-7-2 (lane 3). For control the same extracts were precipitated with MAbs 3063 (lane 4) and 15.75 (lane 5) which were prepared against human MAAs and do not cross-react with fish melanoma, as revealed by IF and IP. M, marker.

sarcoma, but present on cells of a fish melanoma transplant passaged through a nude mouse. In summary our data indicate that the antigens are specifically associated with the neoplastic phenotype of pigment cells, and thus may be considered as MAAs. It also shows that tumor-associated antigens are not restricted to higher vertebrates.

The possibility that the fish MAAs might be melanin is excluded by the fact that they are also present on both the amelanotic melanoma from albinotic fish and the amelanotic melanoma cell line (PSM). In addition, their preferential location on the cell surface argues against melanin being the antigen, since melanin in lower vertebrates is exclusively located inside the cell.

The MAAs specified in the fish melanoma system revealed

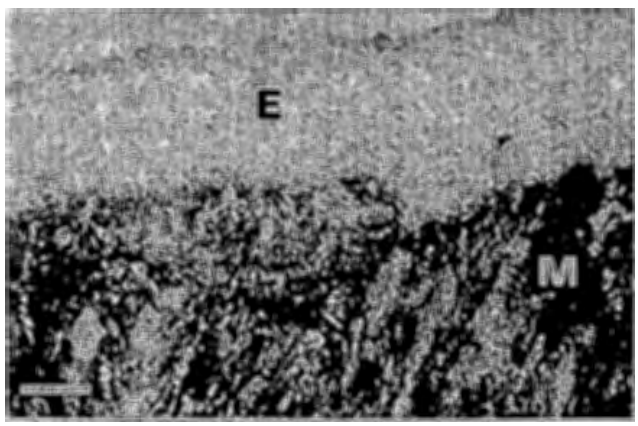

FIGURE 3 – Cryosection of amelanotic malignant melanoma of *Xiphophorus* stained by the immunoperoxidase method using MAb 21-7 as primary antibody and counterstained with hemalum. E = hyperplastic, melanoma-cell free epidermis; M = melanoma. Bar: 50 μm.

TABLE III – AGE-DEPENDENT EXPRESSION OF MELANOMA-ASSOCIATED ANTIGENS ON EMBRYOS AND NEONATES OF *Xiphophorus* FISH, DETECTED BY INDIRECT IMMUNOFLUORESCENCE (IF)

Primary cell culture of embryos from stage	Melanoma-associated antigens	
	21-7	4-7-2
9	NT	—
10	NT	±
11	NT	+
13	+	+
16	+	NT
17	+	NT
18	+	+
23	+	+
24	+	NT
25	+	+
Age of neonates		
1 day	NT	+
2 days	NT	+
4 days	NT	+
10 days	NT	−

+ = positive (80–100% of cells), − = negative; ± = less than 50% of cells positive. NT = not tested.

an expression predominantly restricted to the surface of allogeneic cells. No significant cross-reactivity was found of the 3 MAbs described in this study with a variety of human tumor cells including melanoma. Also, several MAbs generated against human malignant melanoma cells did not react with the fish tumors (Clauss *et al.*, 1990). In investigations on mouse melanoma (Fidler and Kripke, 1977; Fidler, 1978) and human melanoma (Houghton *et al.*, 1981; Woodruff, 1983; Reisfeld, 1985) MAAs are described which are also expressed on xenogeneic mammalian cells. This discrepancy might, however, be a consequence of the large phylogenetic distance between teleost fish and mammals, and it will be interesting to see if the MAAs described in our study are present on pigment-cell tumors of other fish species.

Three cell lines derived from chemically-induced melanomas were stained by all 3 MAbs with only quantitative differences. This suggests that the expression of the MAAs is independent of the etiology of the melanoma, *i.e.*, whether the neoplasm is induced by somatic mutation or is genetically conditioned, as is the case with the primary melanoma and the PSM cell line. A similar situation has been found with the protein product of the c-*src* oncogene in *Xiphophorus* (Schartl *et al.*, 1985).

For the *in vitro* cultured embryo fish cells, heterogeneity in MAA distribution was demonstrated by the 5 established embryo cell lines with reactivities ranging from negative to positive (Table II). A possible explanation for the heterogeneity in MAA expression is that culture conditions might have favored cells with a distinct antigen pattern. This presumption is supported by the finding that all second-passage primary embryo cell cultures gave positive reactions with the 3 MAbs, whereas 4 out of 5 established embryo cell lines did not react with MAb 21-7 and 4-7-2.

In contrast to the established embryo cell lines, all primary embryo cells of all histiotypes expressed the fish MAA, but lost it after reaching a certain age in culture. This may be the result of restriction of expression of the MAAs to cells of a definite phase of embryonic life. Since the MAAs are found on the neoplastically transformed pigment cells and all embryo cells of definite ontogenetic stages, they may represent differentiation or oncofetal antigens. This interpretation is strongly supported by the fact that neonate fish obviously lose the antigenic determinant detected by MAb 4-7-2 at a certain age.

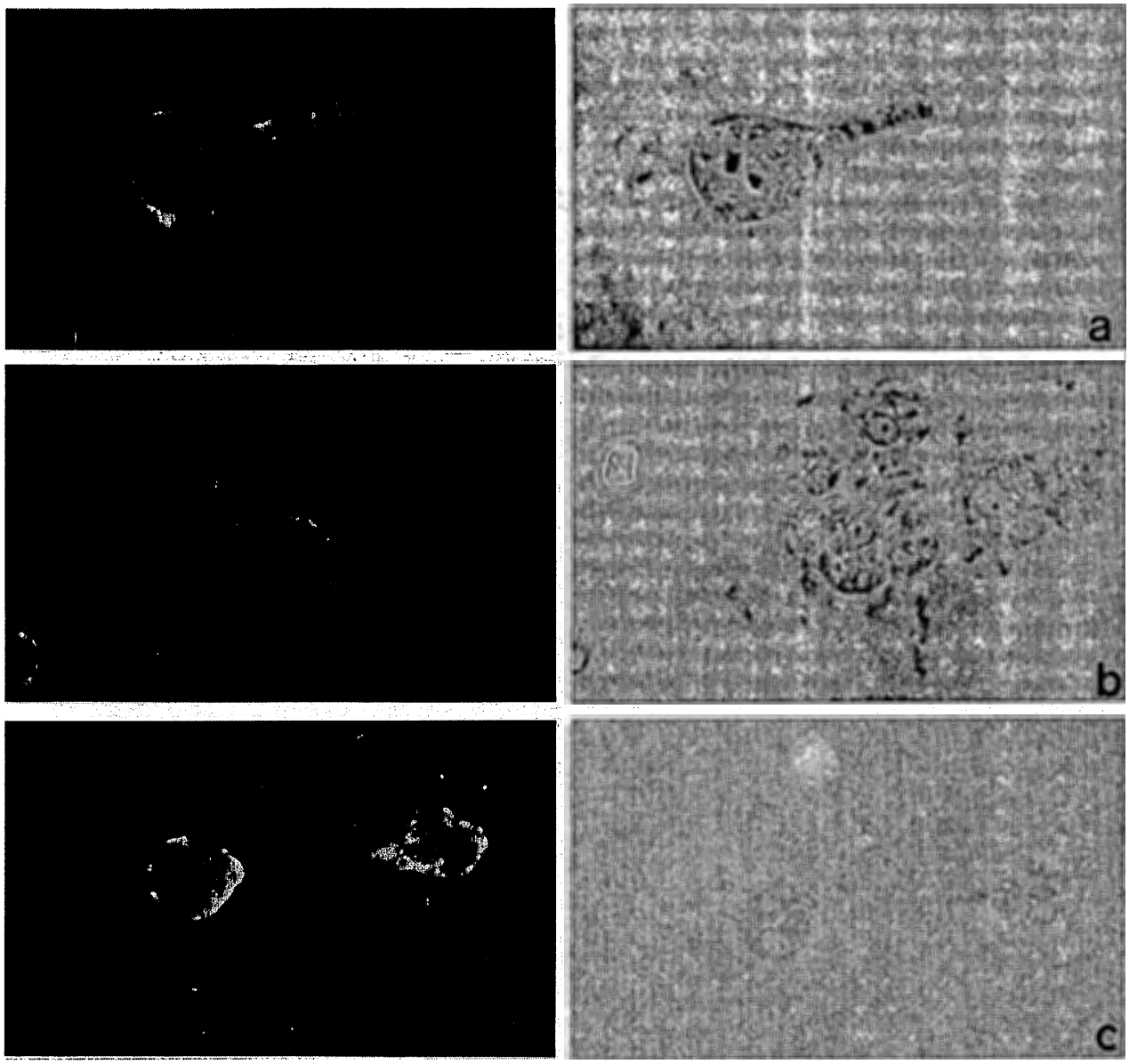

FIGURE 4 – Embryo cells of *Xiphophorus* reacted *in vitro* with MAb 21-7. Left: Immunofluorescence; right: phase-contrast. Cells were derived from primary cultures of embryos stage 22 *(a)*, stage 26 *(b)*, stage 20 *(c)*. Note: post-mitotic cells in *c*.

However, these oncofetal antigens do not specify processes specifically restricted to the pigment-cell lineage.

In human melanoma, several MAAs have been identified as differentiation antigens (*e.g.*, Houghton *et al.*, 1982; Imai *et al.*, 1982; Brüggen and Sorg, 1983; Houghton, 1984) or oncofetal antigens (Herlyn *et al.*, 1980; Garrigues *et al.*, 1982; Thompson *et al.*, 1982; Liao *et al.*, 1985). Steplewski *et al.* (1982) have suggested that in some cases MAAs could be interpreted as the result of over-expression of differentiation antigens on melanoma cells.

The oncofetal antigens recognized by MAbs 21-7 and 4-7-2 demonstrated a qualitatively identical distribution pattern on both primary cells and *in vitro*-cultured cells. In SDS-PAGE analysis both MAbs precipitate a protein duplet of approximately 130 and 135 kDa. This may be explained by the presence of 2 different modified forms of the MAAs (*e.g.*, glycosylation). The identical molecular weight of the precipitated proteins suggests that the antigenic determinants recognized by both MAbs may represent different epitopes on the same molecule or that the two MAbs recognize the same epitope. The different reactivity pattern obtained with MAb 2-18 indicates that this antibody recognizes a different antigen, which was also confirmed by immunoprecipitation studies (Clauss *et al.*, 1990).

Although expression of the MAAs during ontogenesis of *Xiphophorus* is similar to the temporal pattern of kinase activity of the c-*src* oncogene product pp60$^{c\text{-}src}$ (Schartl and Barnekow, 1984), no relationship could be demonstrated, since the 3 MAbs directed against fish melanoma did not show any cross-reactivity with the oncogene product pp60$^{c\text{-}src}$ (A. Barnekow, personal communication). Moreover, the c-*src* mRNA has been localized in normal embryos to cells of neural

origin (Raulf *et al.*, 1989), while the MAbs described here reacted with all embryonic cells regardless of their origin, but not with normal adult brain, where again pp60$^{c\text{-}src}$ is abundant.

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